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# Stress responses in alfalfa (*Medicago sativa* L.). 15. Characterization and expression patterns of members of a subset of the chalcone synthase multigene family

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*Key words:* chalcone synthase (CHS) genes, alfalfa (*Medicago sativa* L.), elicitor, *Phoma medicaginis*

## Abstract

We have identified five different full length chalcone synthase (CHS) cDNA clones from a cDNA library produced from transcripts isolated from an elicitor-treated alfalfa cell suspension culture. Nucleotide sequence similarity between the clones varied from 88–93%. Oligonucleotides based on divergent sequences in the 5′-untranslated regions of the clones could distinguish individual genes, or groups of genes, and their corresponding transcripts. Developmentally regulated expression of the CHS transcripts was predominantly in roots and root nodules; other unidentified members of the CHS gene family are expressed in stems, leaves and nodules. One of the CHS transcripts was strongly expressed in floral tissue. All the CHS transcripts studied were induced in elicitor-treated cell suspension cultures. Transcripts were also induced in roots in response to wounding or spraying with various elicitors, and in leaves infected with *Phoma medicaginis* (but not in wounded leaves). The induction kinetics of CHS2 transcripts were more rapid and/or transient than those of other members of the CHS family in $CuCl_2$-treated roots and *Phoma*-infected leaves. The results are discussed in terms of the evolution and functions of the CHS gene family in legumes.

## Introduction

Chalcone synthase (CHS, EC 2.3.1.74) catalyzes the first reaction specific for the formation of flavonoid compounds, namely the condensation of one molecule of 4-coumaroyl CoA (derived from the central phenylpropanoid pathway) with three molecules of malonyl CoA [15]. CHS is under complex developmental and environmental regulation, consistent with the multiple roles of flavonoid-derived compounds in plants [22]. In addition to their long ascribed functions as UV protectants and insect-attracting flower pigments, flavonoids have also been proposed to act as

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers L02901 (CHS1), L02902 (CHS2), L02903 (CHS4), L02904 (CHS8), L02905 (CHS9).

physiological inhibitors of auxin transport [26]. In legumes, flavonoids play important roles in interactions with microorganisms; as inducers of the nodulation (*nod*) genes of symbiotic *Rhizobium* (flavones and chalcones) [35], stimulators of rhizobial growth (5-hydroxyflavonoids) [24], and antimicrobial phytoalexins (isoflavones, isoflavans and pterocarpans) [15].

Chalcone synthase genes have been cloned from a wide range of plant species, including bean [40], pea [23], soybean [1, 2, 17], parsley [22], tomato [37], Antirrhinum [36], *Petunia* [31], *Arabidopsis* [18], mustard [4], *Matthiola* [16], maize [21] and barley [39]. Generally, the legumes appear to possess families of from approximately 3 (pea) to up to 8 CHS genes (bean, soybean). Most non-legume species examined to date have less than 3 CHS genes, a notable exception being *Petunia* [32]. The chromosomal organization of CHS genes in *Petunia* suggests that the gene family in this organism may have arisen by evolutionarily recent gene duplication events [32], and more than one of the CHS genes of bean has been shown to be linked to another CHS gene on the same $\lambda$ clone [40]. The possession of multiple CHS genes is not essential for a plant's survival, as *Arabidopsis*, *Antirrhinum majus* and parsley all contain a single CHS gene.

Different members of a plant's CHS gene family may exhibit different tissue-specific expression patterns [17, 30] and differential responsiveness to external signals such as light [30, 31, 40], wounding [40] or microbial infection [13, 40]. It is not understood whether these different expression patterns are causally related to the accumulation of different flavonoid end-products in different tissues or in response to different stimuli; the multiplicity of flavonoid functions in legumes makes this question particularly pertinent in these species.

We have chosen alfalfa (*Medicago sativa*) as a model system to study the regulation and functions of the flavonoid pathway. Alfalfa plants and cell cultures produce characterized isoflavonoid phytoalexins on infection [20, 33] or treatment with microbial elicitors [14], and roots and seeds secrete characterized *nod* gene inducing flavones and chalcones [24, 35]. Elicitation of phytoalexins is preceded by the production of transcripts encoding several enzymes of the central phenylpropanoid and isoflavonoid pathways, including CHS [14]. Two-dimensional gel analysis of *in vitro* translated RNA from elicited alfalfa cell suspension cultures indicates the presence of at least 6 elicitor-inducible CHS isopolypeptides [9], suggesting that CHS may exist as a multigene family in alfalfa. We now report the isolation and characterization of 5 distinct but closely related CHS cDNA clones whose transcripts are present in alfalfa cell suspension cultures. We describe the expression patterns of this subset of alfalfa CHS genes in cell cultures and whole plants, in relation to both tissue specificity and inducibility by wounding, elicitors and infection. The transcripts are primarily root-specific, but are inducible in leaves upon fungal infection; one specific transcript is strongly expressed in floral tissues.

## Materials and methods

### *Growth, elicitation and infection of plant material*

Alfalfa (cv. Apollo) cell suspension cultures were initiated and maintained in 50 ml of SH medium as described previously [28]. Following the sixth passage, the cells were grown for 4 days before treatment with baker's yeast elicitor at a final concentration of 50 $\mu$g glucose equivalents per ml. At various time points, cells from elicited and water-treated control cultures were harvested by filtration, frozen in liquid nitrogen and stored at −80 °C until use.

*Medicago sativa* cvs. Apollo and Regen SY were grown under greenhouse conditions. Tissue samples were obtained from healthy plants 8–10 weeks after planting. Floral tissue was harvested at three different developmental stages: unopened flower buds, partially open flowers, fully open flowers. Leaves were subjected to wounding by cutting them into 4 mm long pieces. Cut leaves were placed in Petri dishes containing filter paper soaked in sterile 5 mM potassium phosphate buffer (pH 5.5). The samples were kept in the greenhouse and subsequently harvested, blotted

dry between filter paper and frozen in liquid nitrogen.

*Phoma medicaginis* was grown on V-8 agar; the spores were washed off the sporulating mycelium in 0.1% (w/v) SDS and were used immediately. Excised trifoliate leaves were dipped into the fungal spore solution ($> 10^{10}$ spores/ml), transferred to wet filter paper in Petri dishes and incubated at 25 °C. Control treatments consisted of dipping the leaves in 0.1% SDS only.

Sterile roots were obtained by germinating alfalfa seeds in inverted Petri dishes. Seeds were surface-sterilized by shaking for 5 min in 0.5% (w/v) SDS, 15 min in 70% (v/v) ethanol and 20 min in 20% (v/v) Chlorox bleach. The seeds were washed five times in sterile water. Approximately 100 seeds were placed on 1.5% (w/v) agar in Petri dishes (30 mm × 100 mm) and fixed by 1 ml 0.7% (w/v) top agar. The inverted plates were incubated for approximately 50 h at 25 °C in the dark, until the roots attained lengths of about 2 cm. These roots were sprayed with 2 ml elicitor or water and further incubated as above. Root samples were cut off above the agar surface, frozen in liquid nitrogen and stored at −80 °C. Sterile roots subjected to wounding were cut into 4 mm pieces and incubated in the dark on 5 mM potassium phosphate buffer, pH 5.5.

*Preparation of elicitor solutions*

*Rhizobium meliloti* 102F51 was grown as described elsewhere [11] and *Erwinia chrysanthemi* was grown on King's medium B [38]. Bacterial cultures (containing ca. $10^{10}$ cfu) were centrifuged, resuspended in sterile distilled water, sonicated for 3 min, centrifuged, and the supernatant filter-sterilized through a 0.45 μm nitrocellulose membrane. Baker's yeast elicitor was prepared as described elsewhere [42].

*Synthetic oligonucleotides*

Unique sequences were chosen from the 5'-untranslated regions of the CHS cDNA clones and synthetic oligonucleotides prepared complementary to these sequences. These were as follows: oligonucleotide 2, 5'-CTTTGGTAGC-AAATAAAATG-3' complementary to CHS2; oligonucleotide 10, 5'-AAGTAAAGTACTGT-AATTTG-3', complementary to CHS9 and 10; oligonucleotide 78, 5'-CAGACACACTAACC-ATCTTG-3', complementary to CHS7 and 8; oligonucleotide 1510, 5'-GCAATGATATGG-TAGGTTGG-3' complementary to CHS1, 5, 9 and 10. The oligonucleotide CONI, 5'-ACCCT-TGTTGGTACATCATG-3' was complementary to a conserved part of the coding regions of all the alfalfa CHS cDNAs. All oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer.

*DNA and RNA extraction, blotting and hybridization*

Total DNA was extracted from leaves as described [12, 27], cut with restriction enzymes and separated on a 1.2% agarose gel. Blotting to MSI Magnacharge membranes followed the manufacturer's instructions (Micron Separations, Westborough, MA). Slot blots of plasmid DNA and *in vitro* transcribed RNA were prepared using a Schleicher & Schuell (S&S) manifold apparatus according to the S&S manual.

Total RNA was isolated from cell suspension cultures and plant tissue as previously described [7]. Glyoxal gel electrophoresis was used to size-fractionate RNA. Blotting of RNA to MSI membranes and all hybridization and washing procedures were carried out according to MSI instructions. The *Eco* RI insert of CHS2 was random prime-labeled as described [19]. The insert (1.27 kb) was hybridized to RNA and DNA blots overnight at 65 °C in 5× Denhardt's solution, 6× SSPE, 0.5% (w/v) SDS, 100 μg/ml denatured salmon sperm DNA. Membranes were washed up to a final stringency of 15 min at 65 °C in 0.1× SSC, 1% SDS. Oligonucleotides were labeled using T4 polynucleotide kinase as described elsewhere [41]. Oligonucleotides were hybridized overnight at 35 °C in 6× SSPE, 1%

SDS. The washing conditions differed according to the oligonucleotides. For oligonucleotides 2 and 10, the conditions were 2 × 10 min in 6 × SSPE at room temperature, 5 min in 6 × SSPE, 1% SDS at room temperature, 2 min in 6 × SSPE, 1% SDS at 38 °C and 1 min in 6 × SSPE, 1% SDS at 40 °C as a maximum stringency. Hybridizations with oligonucleotides 78, 1510 and CON1 were further washed for 2 × 2 min in 6 × SSPE, 1% SDS at 42 °C and for 1 min in 6 × SSPE, 1% SDS at 46 °C.

*cDNA library construction*

A cDNA library was prepared from poly(A)$^+$ RNA, from alfalfa (cv. Apollo) cell cultures isolated at 2, 3 and 4 h after exposure to elicitor from *Colletotrichum lindemuthianum* [9], in λZAPII according to the manufacturer's instructions (Stratagene).

*DNA sequence analysis*

Double stranded DNA was sequenced by the dideoxy chain termination method using both synthetic oligonucleotide primers and deletion strategies (exonuclease III nested deletions and the deletion of a *Kpn* I fragment at the 5′ end of the CHS cDNA). Sequence analysis was carried out manually using a United States Biochemicals sequencing kit or on an automated Applied Biosystems 373A Sequencer using *Taq* polymerase.

In vitro *transcription*

Alfalfa CHS clones were cut with *Kpn* I and subsequently purified by phenol/chloroform extraction and ethanol precipitation. *In vitro* transcription essentially followed the protocol described in Sambrook *et al.* [41]; the reaction mix was incubated with T7 RNA polymerase for 30 min at 37 °C and the DNA template was removed by a 30 min treatment with 20U RNase-free DNase.

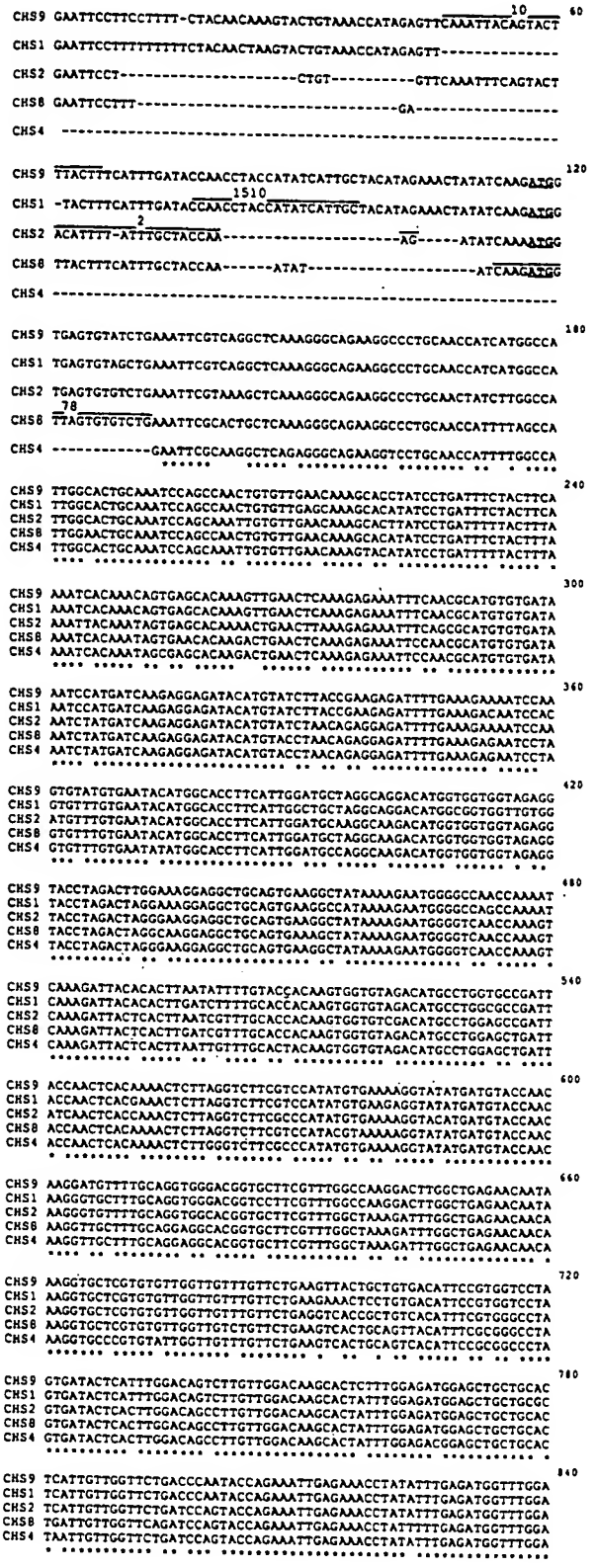
**Results**

*Isolation of CHS cDNA clones*

A cDNA library was prepared in λZAP II from poly(A) RNA isolated from an alfalfa (cv. Apollo) cell suspension culture 2, 3 and 4 h after exposure of the culture to a fungal elicitor [9]. The library (200000 plaques) was screened at low stringency using the full-length CHS17 cDNA from bean [40], and a large number of positive clones were detected, of which 10 were taken through 3 rounds of plaque purification. After autoexcision into pBluescript, Southern analysis revealed that 9 out of the 10 clones contained near full-length inserts. Preliminary 5′ and 3′ end sequencing of these 9 clones indicated that 5 of the clones were indeed full-length, and the others lacked no more than 20 bp of the 5′-coding region. Restriction and partial sequence analysis revealed that CHS1 and 5 corresponded to the same transcript, as did the pairs CHS4/6, CHS7/8 and CHS9/10. We had therefore isolated 5 apparently distinct CHS cDNAs represented by the clones CHS 1, 2, 4, 8 and 9.

*Sequence analysis of CHS cDNAs*

The nucleotide sequences of CHS 1, 2, 4, 8 and 9 were determined and are presented in Fig. 1. CHS4 was truncated 16 nucleotides downstream of the ATG start codon. At the nucleotide level, CHS2 was 78% identical to the bean CHS17 clone used to screen the library. The individual alfalfa CHS clones were more closely related to one another than was any one of them to the bean CHS. There was considerable sequence identity, not only in the coding regions but also in the 5′- and 3′-untranslated regions. The major differences upstream of the ATG start codon were deletions and insertions rather than significant stretches of nucleotide dissimilarity. All 5 cDNAs used TAG as the stop codon. The lengths of the 3′-untranslated regions were 135, 30, 131, 139 and 124 nucleotides for CHS 1, 2, 4, 8 and 9 respectively. The first 120 nucleotides of the 3′-



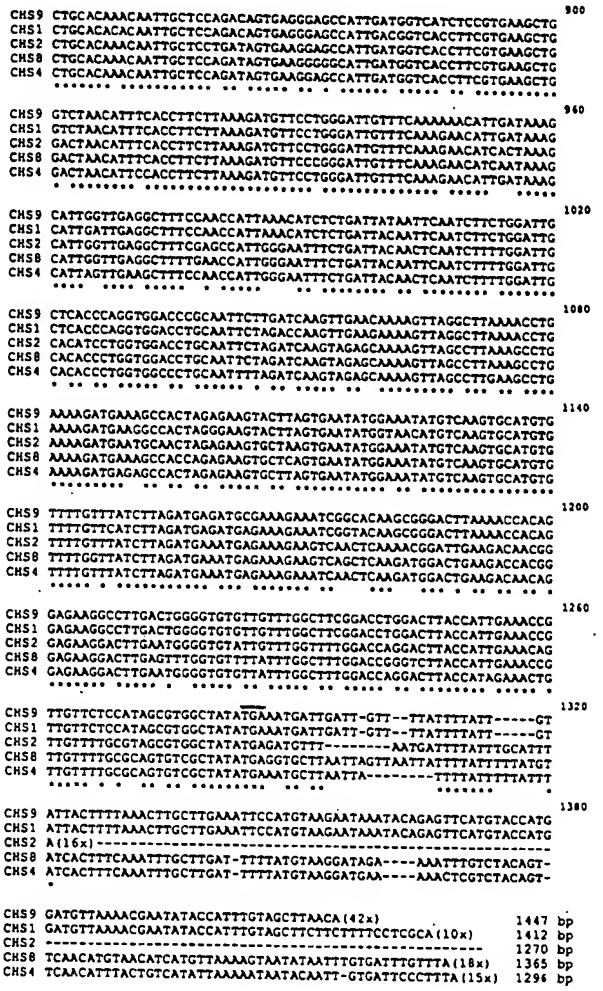


*Fig. 1.* Nucleotide sequence alignments of five alfalfa CHS cDNA clones. Identical nucleotides are marked by *, and gaps in the alignment by –. The translation initiation site is in bold and underlined. Bars above the secluences in the 5′-untranslated or 5′ open reading frame regions denote synthetic oligonucleotides designed to partially distinguish between the individual CHS transcripts (10, 1510, 2, 78). Numbers at the 3′ ends (16×, etc.) refer to the number of A residues present in the cloned poly(A) tail.

untranslated regions of CHS1 and CHS9 were identical and, excluding an 8 nucleotide deletion, the 3′-untranslated region of CHS4 was 87% identical to that of CHS8.

A comparison of the deduced amino acid sequence of alfalfa CHS2 with bean CHS17 and the CHS consensus sequence [36, 40] is shown in Fig. 2. Alfalfa CHS2 deviated more from the consensus than did the bean CHS (16 amino acids different, as compared to 4 for bean). Of the previously published CHS sequences examined, the *Arabidopsis* CHS [18] was most divergent from alfalfa CHS2 at both the DNA and amino acid sequence levels (66% and 81% respectively). Comparison of the deduced amino acid sequences from all 5 alfalfa CHS clones (Fig. 3) showed extensive sequence conservation, although many of the amino acid differences between the individual CHS cDNAs were not conservative changes. The calculated isoelectric points of the 5 CHS proteins ranged from 5.7 to 6.1, within the range observed for CHS isopolypeptides resolved by two-dimensional SDS-PAGE: IEF following *in vitro* translation of mRNA from elicitor-induced alfalfa cell suspension cultures [9].

*Design of oligonucleotides to discriminate between alfalfa CHS transcripts*

Four 20-mer oligonucleotides were synthesized based on sequence differences between four of the alfalfa CHS cDNA clones. Oligonucleotides 10, 1510 and 2 were complementary to sequences within the 5′-untranslated regions of CHS 9/10, 1 and 2 respectively, whereas oligonucleotide 78 was complementary to a region of CHS8 overlapping the ATG start codon and extending into the open reading frame (Fig. 1). A fifth 20-mer oligonuceotide, CON1, was complementary to the region encoding amino acids 158–163 (MMYQQG) (with one extra nucleotide at each end) in all the CHS clones.

The sequence specificity of the 5 oligonucleotides was tested by dot blot analysis in which they were hybridized to immobilized cDNA, or

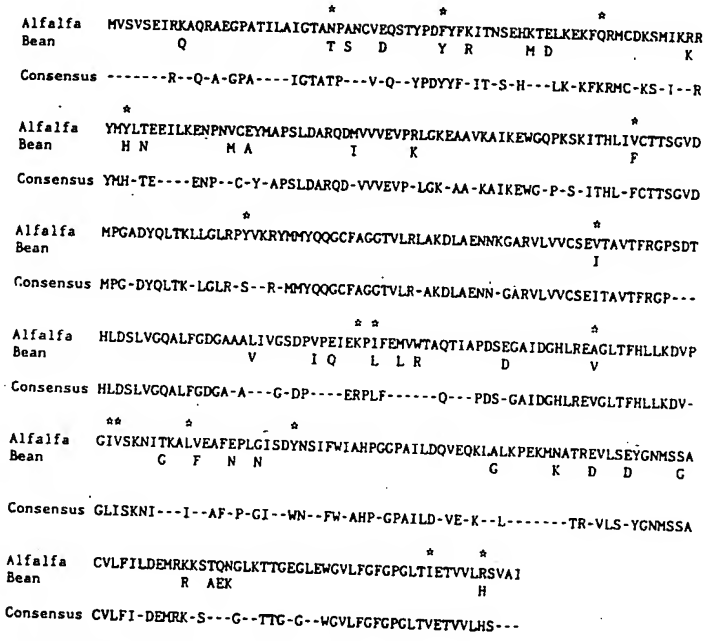



*Fig. 2.* Deduced amino acid sequence comparison between alfalfa CHS2, bean CHS17 [40] and the CHS consensus sequence derived from amino acid sequences of the enzyme from *Antirrhinum majus*, *Hordum vulgare*, *Magnolia liliiflora*, *Petunia hybrida*, *Ranunculus acer*, *Petroselinum hortense* and *Zea mays* [36]. Only those amino acids in the bean sequence which differ from the alfalfa sequence are shown. A * indicates where the alfalfa sequence differs from the consensus.

```
     1                                                                                                    
CHS9 MVSVSEIRQAQRAEGPATIMAIGTANPANCVEQSTYPDFYFKITNSEHKVELKEKFQRMCDKSMIKRRYMYLTEEILKENPSVCEYMAPSLDARQDMVVV 100
CHS1 ----A---------------------------------------------------------------------------------------------
CHS2 --------K----------L-----------------------------T-------------------------------N-----------------
CHS8 --------T----------L-----------------------------T-------------------------------------------------
CHS4       --K----------L-----------------------------T-------------------------------------------------
           ** ********** ***************************** ************************** ** ********* ***** **
     101                                                                                                  
CHS9 EVPRLGKEAAVKAIKEWGQPKSKITHLIFCTTSGVDMPGADYQLTKLLGLRPYVKRYMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEVTAVTFRG 200
CHS1 V---------------------------------------------------------------------------------------------
CHS2 E---------------------------V-----------------------------------------------------------E-P------
CHS8 E---------------------------V-----------------------------------------------------------------
CHS4 E---------------------------V-----------------------------------------------------------------
     ************************** ******************** ************************************** * *****
     201                                                                                                  
CHS9 PSDTHLDSLVGQALFGDGAAALIVGSDPIPEIEKPIFEMVWTAQTIAPDSEGAIDGHLREAGLTFHLLKDVPGIVSKNIDKALVEAFQPLNISDYNSIFW 300
CHS1 ----------------------------------------H----------------------------------------------------------
CHS2 ---------------------------V-----------------------------------------------------------------------
CHS8 ---------------------------V----------------------G-----------------------N-------E--G---------
CHS4 ---------------------------V-----------------------------------------------------------G---------
     ************************** ************ ******** ************************************ ******* ** *********
     301                                                                                                  
CHS9 IAHPGGPAILDQVEQKLGLKPEKMKATREVLSEYGNMSSACVLFILDEMRKKSAQAGLKTTGEGLDWGVLFGFGPGLTIETVVLHSVAI 389 pI   5.8
CHS1 ------------E-----------------------------------------V---------------------------------- pI   6.1
CHS2 ----------------A------N---------------------------T-N---------E-----------------R---- pI   5.9
CHS8 ----------------A-------------------------------------D---------EF----------------R---- pI   5.7
CHS4 ----------------A------R---------------------------T-D---------E-----------------R---- pI   5.9#
     ************** ** ****** ************************** * ********* **************** **** 
```

*Fig. 3.* Deduced amino acid sequence alignments of alfalfa CHS cDNA clones (compared to CHS 9). Amino acids conserved in all 5 sequences are marked with *. Deduced isoelectric points are given after each sequence. The pI of the incomplete CHS 4 (#) was calculated assuming that the six N-terminal amino acids are the same as for the other CHS clones.

*in vitro* transcribed RNA, corresponding to all 9 of the full-length or near-full-length CHS clones initially isolated (Fig. 4). Oligonucleotide 2 only hybridized to CHS2 cDNA or its transcripts, whereas oligonucleotide 10 specifically recognized the two presumed identical CHS9 and 10 clones/transcripts. Oligonucleotide 78 recognized the presumed identical CHS7 and 8 clones/transcripts; it weakly hybridized to CHS1, 2, 5, 9 and 10 cDNAs, but did not recognize their transcripts. Unlike oligonucleotides 2, 10 and 78, oligonucleotide 1510 was not specific for one particular class of CHS transcript; it recognized the cDNAs/transcripts of CHS1, 5, 9 and 10. As predicted, the conserved CON1 oligonucleotide recognized all members of the CHS family analyzed.

As the 5′ untranslated region of CHS4 is missing, the lack of hybridization of the 2, 10, 78 and 1510 oligonucleotides to CHS4 in Fig. 4 does not imply that the full length transcript will not be recognized. Thus, in the following results and discussion, the possibility that CHS4 transcripts are being detected by one, several or all of the oligonucleotide probes must be borne in mind.

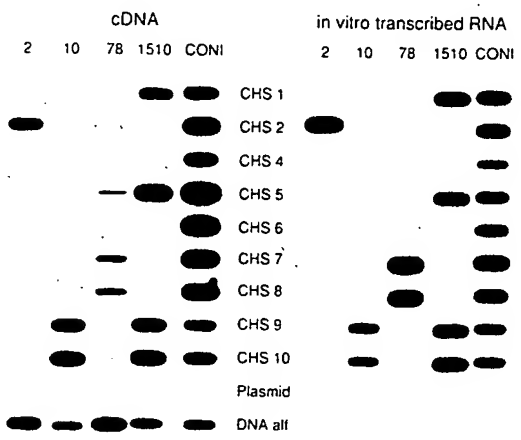



*Fig. 4.* Specificity of oligonucleotides constructed to recognize regions of different members of the alfalfa CHS gene family. Left-hand lanes: alfalfa CHS clones 1 through 10 (1 ng), pBluescript plasmid DNA (1 ng) and alfalfa genomic DNA (10 $\mu$g) were immobilized on slot blots and hybridized with $^{32}$P-labeled 2, 10, 78 and 1510 oligonucleotides (See Fig. 1). CON1 is a 'consensus' oligonucleotide, corresponding to nucleotides 507 to 526 of the CHS2 cDNA, which recognizes all the cDNA clones isolated in this work. Right-hand lanes: an identical slot blot, but containing immobilized transcripts corresponding to CHS1 through 10 synthesized *in vitro* from pBluescript clones using T7 polymerase (0.1 $\mu$g/slot).

*Genomic organization of alfalfa CHS*

Southern blot analysis of alfalfa genomic DNA, using the full-length CHS2 cDNA as probe (Fig. 5), indicated that CHS is encoded by a relatively complex multigene family in alfalfa. The complexity of the Southern hybridization pattern was somewhat greater than that previously observed for bean (*P. vulgaris*), which possesses 7 different CHS genes [40]. A number of polymorphisms were observed between the CHS restriction fragment lengths from two tetraploid alfalfa cultivars Apollo and Regen SY (the latter being a highly regenerable line).

Hybridization of *Hin*d III-digested alfalfa genomic DNA with specific oligonucleotides revealed some genomic fragments which were specifically recognized by individual oligonucleotides, plus higher-molecular-weight fragments which seemed to be recognized by more than one oligonucleotide (data not shown). However, the resolution of the higher-molecular-weight hybridizing fragments was poor in several replicated experiments, and it was not possible to conclude how many copies of each gene were present in the alfalfa genome.

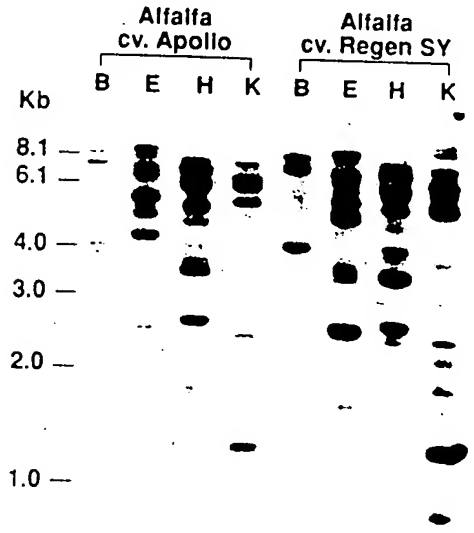



*Fig. 5.* Southern blot analysis of alfalfa CHS genomic sequences. Genomic DNA (15 μg) from alfalfa cvs. Apollo or Regen SY was digested with *Bam* HI (B) or *Eco* RI (E), *Hin*d III (H) or *Kpn* I (K), electrophoresed in a 1.2% agarose gel, blotted and probed with the full-length CHS2 cDNA.

*Expression of CHS transcripts in alfalfa cell suspension cultures*

Exposure of an alfalfa cell suspension culture to yeast elicitor resulted in a rapid and transient increase in CHS transcripts, as determined by northern blot analysis of total RNA probed with the full-length CHS2 cDNA, which does not discriminate between different members of the CHS gene family. Maximum transcript levels were attained around 4 or 5 h after elicitation, although a large increase had occurred as early as 1 h after elicitation (Fig. 6). The kinetics of appearance of specific CHS2 and CHS10 transcripts were broadly similar to the pattern revealed by the nonspecific cDNA probe, although the accumulation of CHS2 transcripts appeared more transient than that of the total CHS population. Indeed, in

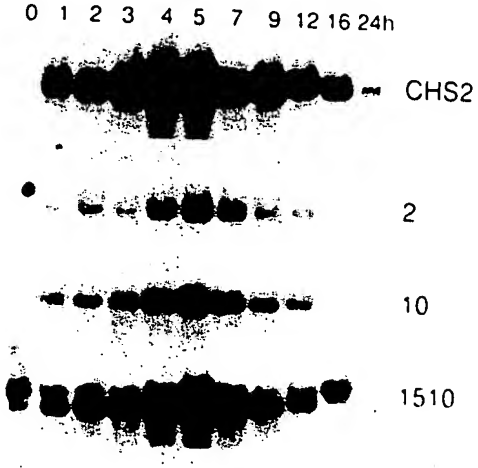



*Fig. 6.* Appearance of CHS transcripts in alfalfa cell suspension cultures following exposure to yeast elicitor (50 μg glucose equivalents per ml). Total RNA was isolated from cultures at various times post-elicitation, and subjected to northern blot analysis (10 μg/lane). Blots were probed with the full length CHS2 cDNA and oligonucleotides 2, 10 and 1510. No signal was observed in unelicited control cultures. Blots were exposed to X-ray film for 2 days (1510), 3 days (2 and 10) and 16 h (CHS2). The blot for the oligo 10 hybridization was stripped and re-probed with CHS2.

later experiments (see tissue culture controls in Figs. 8 and 9), CHS2 transcripts were very low, or not present at all, 7 h after elicitation. The slightly different kinetics of appearance of the transcripts detected by the CHS2 and CHS10 oligonucleotides, and the fact that a single transcript of 1.6 kb was observed in both cases, further supports the conclusion that oligonucleotides 2 and 10 are transcript-specific. The oligonucleotide 1510, which recognizes CHS 1, 5 and 10 transcripts, hybridized to an elicitor-inducible transcript of 1.6 kb and a slightly higher $M_r$ transcript which may be unrelated to CHS, but which could also be either an unspliced transcript of CHS 1, 5 or 10, or a transcript corresponding to an uncharacterized CHS gene family member. It should be noted that, in Fig. 6, the blots probed with oligonucleotides were exposed for 2–3 days, whereas the final probing with the full length CHS2 cDNA was only exposed for 16 h; thus, the data do not reveal relative transcript levels. The lack of signal at zero time in the blot probed with the full length CHS2 cDNA might reflect loss of RNA due to re-probing the blot. An increase in CHS transcripts was not observed in control, unelicited cultures (data not shown).

*Tissue-specific expression of CHS transcripts*

Northern blot analysis of total RNA from various organs and tissues of mature alfalfa plants indicated that, as a whole, the CHS gene family was primarily expressed in the lower parts of the plant (roots, nodules, and lower stems). Moderate expression was also seen in upper stems and petioles, with very low expression in leaves and mature flowers (Fig. 7A). Specific CHS 2, 10 and 7 transcripts were, in contrast, expressed exclusively in roots and, to a much lesser extent, in nodules.

As CHS is often highly expressed in flowers

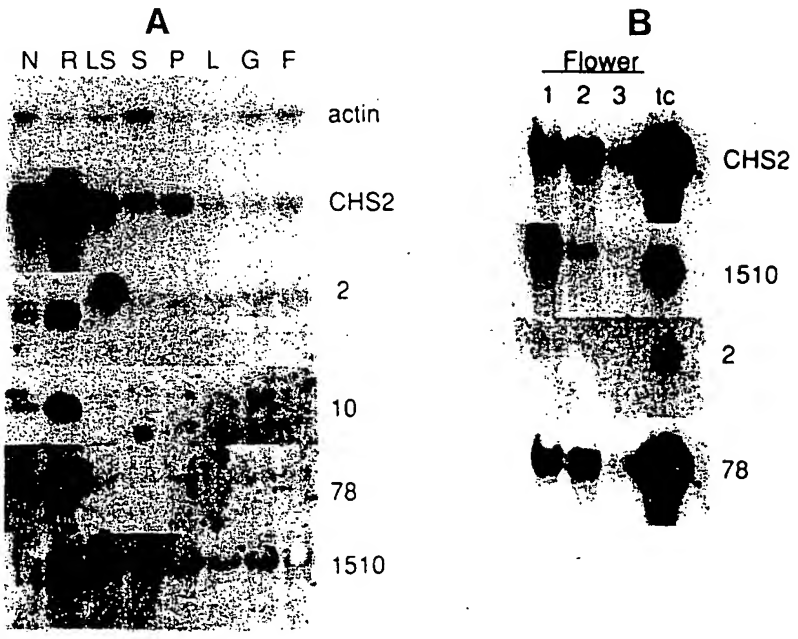



*Fig. 7.* Tissue-specific expression of alfalfa CHS genes. A. Northern blot analysis of total RNA (10 μg/lane) from nodules (N), mature roots (R), lower stems (LS), upper stems (S), petioles (P), leaves (L), growing points (G) and mature flowers (F) probed with the full-length CHS2 cDNA, the 2, 10, 78 and 1510 oligonucleotides, and the soybean actin sAc3 genomic clone . B. Northern blot analysis of total RNA (10 μg/lane) from unopened flower buds (1), partially open flowers (2), fully open flowers (3) and yeast elicitor-induced cell suspension culture (tc), probed with the CHS2 cDNA and the 1510, 2 and 78 oligonucleotides. Blots probed with oligonucleotides were exposed to X-ray film for 4–6 days, and with CHS2 for 24 h.

[23, 30, 31], we examined alfalfa floral tissue, at 3 stages of development, for CHS transcripts (Fig. 7B). Transcripts were abundant at early developmental stages, but were nearly absent from the mature flower. However, 1.6 kb CHS 1, 5 or 10 transcripts or CHS2 transcripts were not present at any stage of floral development; only the high $M_r$ transcript hybridizing to the 1510 oligonucleotide was observed. This transcript appeared to be developmentally regulated in a similar manner to the 1.6 kb CHS transcripts, suggesting that it might encode a larger CHS transcript. CHS7/8 transcripts were expressed in floral tissue in a similar manner to the pattern revealed by hybridization to the non-discriminating CHS2 cDNA. As the CHS2 oligonucleotide only weakly hybridized to elicited cell culture RNA in Fig. 7B, it is not possible to rule out that a low level of CHS2 transcripts may be present in floral tissue.

*Induction of CHS transcripts in roots and leaves by elicitation, wounding and infection*

By germinating alfalfa seeds in inverted Petri dishes, it was possible to obtain young, sterile root tissue which could be readily sprayed with elicitor solutions. Figure 8 shows the results of such an experiment in which roots were exposed to water (control), yeast elicitor, the abiotic elicitor $CuCl_2$, or extracts from the pathogenic bacterium *Erwinia chrysanthemi* or the symbiotic bacterium *Rhizobium meliloti*. All treatments significantly increased total CHS transcript levels (measured with the CHS2 cDNA) above those observed in control roots, and to levels approaching those seen in yeast elicitor-treated cell suspension cultures. Note that the transcript levels observed in untreated young, Petri-dish-grown roots were much lower than in mature roots from 8–10 week greenhouse-grown plants (Fig. 7A). The three biotic elicitors (*Erwinia* extract, yeast elicitor and *Rhizobium* extract) induced a rapid response after which transcript levels then decayed as a function of time; in contrast, there was a gradual increase in transcript levels up to 18 h after elicitation in roots treated with the abiotic elicitor $CuCl_2$. The same pattern was observed on probing the blot with the 1510 and 10 oligonucleotides. However, CHS2 transcripts behaved in a different manner, being rapidly induced by $CuCl_2$ and then declining.

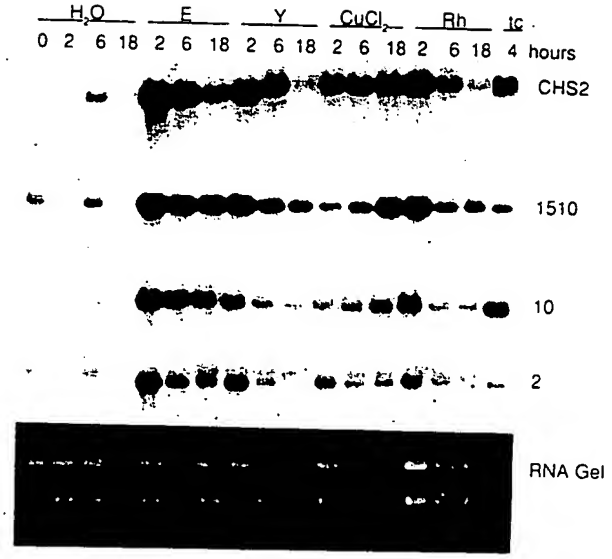



*Fig. 8.* Expression of the CHS gene family in young roots in response to biotic or abiotic elicitors. Roots were sprayed with $H_2O$ (control), yeast elicitor (Y, 500 μg glucose equivalents per ml), $CuCl_2$ (50 mM), and extracts from *Erwinia chrysanthemi* (E) and *Rhizobium meliloti* (Rh). Total RNA was extracted from roots incubated for the times shown, and subjected to northern blot analysis (10 μg/lane) with the CHS2 cDNA and the 1510, 10 and 2 oligonucleotides as probes. The right-hand lanes (tc) contain 4 μg of total RNA from alfalfa cell cultures exposed to yeast elicitor for 4 h. The lowest panel shows ethidium bromide staining of the gel prior to northern transfer.

Wounding mature alfalfa leaves by cutting resulted in a significant increase in CHS transcripts (hybridizing to the full-length CHS2 cDNA), which attained maximum levels around 4 h after the stimulus (Fig. 9). However, no transcripts could be detected by the oligonucleotides 1510, 10 or 2 in leaves. A larger and more rapid increase in CHS transcripts was observed in similarly wounded root tissue, and in this case transcripts were detected by the three oligonucleotides. However, CHS2 and 10 transcripts were seen at very low levels at 2 h after wounding. The low level of CHS10-specific transcripts suggests that

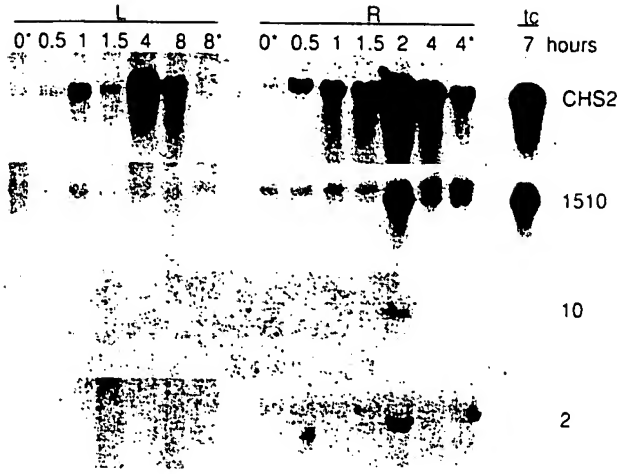



*Fig. 9.* Effects of wounding on expression of the CHS gene family. Total RNA was extracted from leaves (L) or roots (R) that had been wounded by cutting and harvested at the times shown. * Indicates control, unwounded tissue. tc indicates RNA from a yeast elicitor treated cell suspension culture. Northern blots (10 μg RNA/lane) were probed with the CHS2 cDNA and the 1510, 10 and 2 oligonucleotides.

the 1510 oligonucleotide is primarily detecting CHS1 or 5 transcripts in wounded roots.

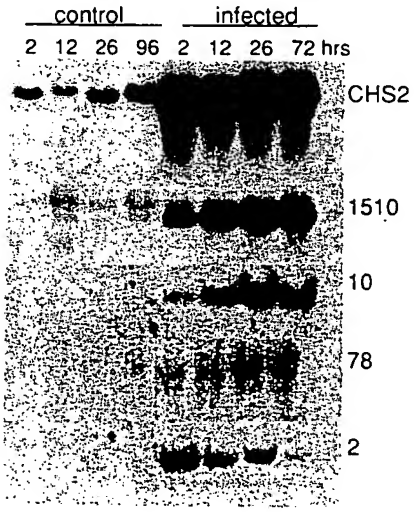



*Fig. 10.* Effects of infection by *Phoma medicaginis* on expression of the alfalfa CHS family in leaves. Excised leaves were dipped in a spore suspension of *P. medicaginis* or $H_2O$ (control) and incubated at 25 °C for the times shown. Northern blots of total RNA (10 μg RNA/lane) were probed with the CHS2 cDNA and the 1510, 78, 10 and 2 oligonucleotides.

Dipping excised alfalfa leaves in a spore suspension of the fungus *Phoma medicaginis* results in the appearance of massive leaf spot symptoms within ca. 48 h. This response was associated with very rapid (by 2 h) induction of CHS transcripts, and maintenance of the elevated transcript level up to 72 h after inoculation (Fig. 10). Control leaves showed a substantially smaller rise in total CHS transcripts, presumably as a response to excision wounding. No transcripts detected by the CHS-specific oligonucleotides were observed in excised control leaves, consistent with the data in Fig. 9. However, *Phoma* infection led to significant increases in transcripts detected by oligonucleotides 1510, 10, 78 and 2, which increased more gradually than 'total' transcripts as a function of time after infection. In contrast, CHS2 transcripts were at their maximum levels 2 h after infection, and then decreased to barely detectable levels by 72 h.

## Discussion

Our data indicate that alfalfa, like other legumes, contains a family of CHS genes. Up to 8 CHS genes have been reported in soybean [17], 6–8 in bean [40], and more than 3 in pea [23], and 5 CHS *in vitro* translation products have been resolved from elicited chickpea cell culture RNA [10]. Southern blots of alfalfa genomic DNA were at least as complex as those for bean, suggesting a minimum of 6 CHS genes in alfalfa. Multiple genes in alfalfa may be explained by allelic variation due to the obligate outbreeding tetraploid nature of the plant. However, in this respect it is interesting to note that we have observed similarly complex CHS genomic hybridization patterns in *Medicago truncatula*, which is strictly self-fertilizing, diploid, and may have a significantly smaller haploid genome size than alfalfa [3] (unpublished results). Genomic Southern hybridization patterns for alfalfa are generally more complex than for *M. truncatula* [3], and alfalfa cv. *Apollo* has been described as exhibiting large within population RFLP variation [6]. It is therefore likely that the apparent complexity of the

alfalfa CHS gene family is not principally due to allelic variability.

The five sequenced alfalfa cDNA clones show considerable sequence identity. In particular, the highly conserved 3′-untranslated regions (especially for CHS1 and 9) suggest that these members of the family may have arisen by gene duplication, as proposed for two CHS subfamilies from *Petunia* [32]. Gene duplication is quite extensive in alfalfa [6]. Published sequences for members of the bean, soybean and *Petunia* CHS families likewise reveal highly conserved coding regions, but in these cases the 3′-untranslated regions were quite divergent [17, 32, 40]. In *Petunia*, members of CHS subfamilies are genetically linked, but the physical distance between the genes is still considerable [32]. The CHS 1 and CHS 2 genes of pea are not clustered [23]. In contrast, both bean and soybean contain CHS genes so closely linked that two genes can be isolated on the same $\lambda$ clone [1, 2, 40]. Our preliminary analysis of alfalfa genomic clones also suggests close physical linkage of some CHS genes in this species (unpublished results).

CHS sequences have been used to construct phylogenetic trees [36]. The alfalfa CHS sequences reported here are more similar to those of other legume CHSs than they are to CHSs from monocots or other dicots. The GC content of the alfalfa CHS coding sequences is low (42% for CHS 2), typical of dicot CHS sequences [36]. The alfalfa CHS 2 differs from the consensus sequence derived from the sequences of CHS from *Antirrhinum majus*, *Hordeum vulgare*, *Magnolia liliiflora*, *Petunia hybrida*, *Ranunculus acer*, *Petroselinum hortense* and *Zea mays* [36] by only 16 out of 389 amino acids. Included in a conserved region of 28 amino acids near the C-terminus of the CHS open reading frame from alfalfa and all other species is the sequence MSSACV, which contains cysteine 341, believed to be an essential feature of the CHS active site [39].

CHS sequences are, overall, much less divergent than sequences encoding phenylalanine ammonia-lyase (PAL), the first enzyme of the phenylpropanoid pathway. Individual members of the PAL gene family in bean show extensive divergence in the N- and C-terminal portions [8]. Expression of these genes leads to the synthesis of PAL isoforms with different kinetic properties [5], presumably reflecting functional specialization. Whether the different CHS enzymes in alfalfa differ in their kinetic properties is a question for future biochemical studies.

The five isolated cDNAs hybridized to transcripts which, under apparent non-stress conditions, are predominantly expressed in root tissues. This is consistent with the observation that the type of cell culture line from which they were isolated (derived from seed germinated directly on callus induction medium) exhibits an isoflavonoid profile typical of that of roots [29; N. L. Paiva, unpublished observations]. CHS transcripts have been shown to be expressed in roots in soybean [17], pea [23] and parsley [30]. The greater overall transcript level present in roots as compared to root nodules observed in alfalfa has also been reported in soybean [17]. In parsley, expression of transcripts from the single CHS gene declines in response to wounding [34], in contrast to the situation in alfalfa. CHS does not appear to be expressed in roots in *Petunia* [34]. One obvious function for CHS expression in legume roots is in the synthesis of the flavonoid derivatives which are secreted into the rhizosphere and which induce the *nod* genes of *Rhizobium*. In alfalfa, these compounds include 4′,7dihydroxyflavanone, 4′,7-dihydroxyflavone and 4,4′-dihydroxy-2′-methoxychalcone [35]. Alfalfa roots also accumulate significant levels of isoflavone and pterocarpan malonyl glycosides [29; N.L. Paiva and M.J. Harrison, unpublished results]. In alfalfa cell suspension cultures, the induction of CHS precedes the accumulation of the pterocarpan phytoalexin medicarpin [10, 14]. In species such as *Petunia*, which do not produce flavonoid derivatives as defense compounds, CHS is not elicitor-inducible [30].

Transcripts corresponding to the alfalfa cDNAs reported here are not present in uninfected leaves. However, northern blot analysis using a full length CHS cDNA as probe indicates that other members of the CHS family are expressed in this tissue. In *Petunia*, the vast majority

of CHS expression occurs in floral tissues, primarily associated with the accumulation of anthocyanin pigments [30, 31]. Alfalfa petals contain delphinidin, petunidin and malvidin 3,5-diglucosides [43]. These pigments accumulate prior to opening of the flower buds, consistent with the transcript expression pattern in Fig. 7B. CHS7/8 was most strongly expressed in floral tissues; it is not clear whether any specific CHS gene products might have a biochemical specialization for the anthocyanin pathway rather than the 5-deoxy-flavone/-flavanone/-isoflavone pathways of roots.

It has been inferred from studies using transcript-specific CHS probes that soybean contains a subset of CHS genes which are specifically expressed during the normal development of root nodules [17]. Our data, in which transcript-specific oligonucleotides possibly do not recognize the total complement of CHS transcripts in alfalfa root nodules, suggest that this may also be true in alfalfa. It has been proposed that expression of CHS in the pea nodule primordium is related to production of flavonoids which function as auxin transport inhibitors [44]. This is supported by the ability of the auxin transport inhibitors *N*-(-1-naphthyl)phthalamic acid and 2,3,5-triiodobenzoic acid to induce pseudonodules in alfalfa in which early nodulin genes are expressed [25]. Different CHS genes are activated in different cell types in ineffective nodules induced by mutant *Rhizobium*, suggesting that if normal symbiosis does not occur, a defense response is activated [44]. Our data suggest that alfalfa roots perceive crude extracts from *Rhizobium* as elicitors, resulting in induction of the root and infection-specific subset of CHS genes. It will be interesting to study the pattern of CHS gene expression in response to the lipo-oligosaccharide nodulation signals of *R. meliloti*.

Although transcripts corresponding to the 5 alfalfa CHS clones are not normally expressed in leaves, they were induced in leaves in response to fungal infection, but not wounding. Thus, some environmental cues can override the tissue-specific expression pattern of alfalfa CHS genes. A similar situation has been reported in bean with respect to infection and UV treatment [40]. The interaction between alfalfa leaves and *Phoma medicaginis* is a compatible interaction, i.e. a successful infection in which disease symptoms develop. In bean, differential expression of CHS genes in compatible and incompatible (hypersensitive resistant) interactions has been reported [40]. In soybean, CHS transcripts accumulated to higher levels in leaves infected with incompatible as compared to compatible strains of the bacterial pathogen *Pseudomonas syringae* pv. *glycinea*, although in roots similar levels of transcripts were observed in response to compatible or incompatible races of the fungus *Phytophthora megasperma* f.sp. *glycinea* [13]. The response to *Phoma* in our experiments was much more rapid than the above responses of bean or soybean to pathogens. It is possible that the *Phoma* spore suspension contains elicitors which induce CHS transcription, since the appearance of CHS transcripts occurred prior to any development of disease symptoms.

Although the 5 alfalfa CHS transcripts share similar tissue-specific expression patterns and are co-induced in response to elicitor, wounding or infection, their induction kinetics differ. In particular, CHS 2 transcripts reach their maximum levels and then decline earlier than do the other transcripts in $CuCl_2$-treated roots and *Phoma*-infected leaves. Similarly, differences in induction kinetics are observed for individual members of the bean CHS family in elicited cell cultures [40]. Future studies will be directed at understanding the molecular basis for the differential expression patterns of the alfalfa CHS gene family, and evaluating whether individual CHS enzyme isoforms exhibit biochemical specialization.

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